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Comparison of two PCR strategies for the detection of bovine papillomavirus

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Bovine papillomavirus (BPV) is a diverse group of double-stranded DNA oncogenic viruses, which have been detected in epithelial lesions and body fluids. Most studies of BPV infection rely on a single method for DNA detection; however the use of any single method or technique may underestimate the true prevalence of this virus. The purpose of this study was to compare two PCR strategies for the detection of BPV in skin lesions and fluids: these involve the use of BPV type-specific and consensus primers. Seventy-two cutaneous lesions, 57 blood samples and 59 semen samples were collected. PCR was used with the FAP consensus primers and BPV type-specific primers (for BPVs 2, 3, 4, 5, 8, 9 and 10), along with sequencing assays, to detect the BPV types. Phylogenetic analysis was carried out by means of the maximum likelihood method. It was found that both FAP and BPV type-specific primer sets could amplify BPV types of DNA in skin lesions, blood and semen samples. However, the BPV type-specific primers were more sensitive than the consensus primers and were able to detect co-infection of BPV in the samples. The consensus primers amplified five BPV types and were more suitable for detecting new putative BPV types. Thus, account should be taken of both PCR primer systems to identify co-infection, the presence of novel viruses, and avoid false-negative results.

1. Introduction

Papillomaviruses (PVs) are a group of double-stranded DNA viruses, which have been identified in a broad range of animal species (Freitas et al., 2011). They are classified in the *Papillomaviridae* family which comprises 29 different genera (Bernard et al., 2010). More than 150 human papillomaviruses (HPV) have been identified while only 13 bovine papillomavirus (BPV) types have been described (Lunardi et al., 2013).

BPV can induce papillomas and fibropapillomas in the skin (Nasir and Campo, 2008) and mucous lesions, which can regress or evolve to malignant lesions, usually when influenced by environmental co-factors. Some BPV types are involved in the urinary bladder (BPV1 and BPV2) and upper digestive tract (BPV4), where they form malignant tumors in cattle (Borzacchiello and Roperto, 2008).

BPV has been detected in non-epithelial sites such as gametes and fluids in recent years (Freitas et al., 2003; Roperto et al., 2008; Diniz et al., 2009; Lindsey et al., 2009; Roperto et al., 2011; Silva et al., 2011), and blood has been hypothesized as a carrier of BPV to various body parts (Freitas et al., 2007; Roperto et al., 2011). In addition, blood and semen have been described as providing a site for BPV gene expression in cattle and horses (Roperto et al., 2008, 2011; Silva et al., 2013).

PVs have been detected and characterized by PCR with consensus primers (Manos et al., 1989; Forslund et al., 1999; Ogawa et al., 2004). Two sets of primers (FAP59/FAP64 and MY09/MY11), which were originally designed from two conserved regions of the HPV L1 gene, are widely used for identifying PVs in both humans and a wide range of animals (Manos et al., 1989; Forslund et al., 1999; Antonsson and Hansson, 2002; Ogawa et al., 2004). About 31 putative new BPV types were detected in bovines by means of these primers (Forslund et al., 1999; Antonsson and Hansson, 2002; Ogawa et al., 2004; Maeda et al., 2007; Claus et al., 2008; Carvalho et al., 2012). Apart from the use of consensus primers, several BPV type-specific primers have been designed for the purpose of identifying specific BPV types (Gaukroger et al., 1991; Bloch et al., 1997; Wosiacki et al., 2005; Brandt et al., 2008; Silva et al., 2011; Carvalho et al., 2012).

Some studies in HPV suggest that the use of just a single method or technique for the detection, may underestimate the true prevalence of this virus (Karlsen et al., 1996; Smits et al., 1995). In cattle, most studies of BPV infection rely on a single method. Since the knowledge of BPV diversity and its prevalence is very important

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for the development of treatment and diagnostic methods, as well as for understanding the evolution and epidemiology of BPV, two PCR strategies for its detection were compared in skin lesions and fluids from Brazilian cattle – the use of BPV type-specific and consensus primers. This report highlights the robustness and weakness of each method and shows the importance of using both of them.

2. Materials and methods

2.1. Skin lesions

A total of 72 skin lesions were collected from beef and dairy cattle on farms in Northeastern Brazil. The whole procedure of collection was performed by a specialized veterinary surgeon and in compliance with international ethical standards for animal welfare.

2.2. Blood samples

Blood samples were collected from 57 cattle from beef and dairy farms in North-East Brazil, which had a high incidence of cutaneous papillomatosis. Three mL of blood was collected by jugular venipuncture using EDTA-containing tubes and 200 μL of total blood was used for DNA extraction.

2.3. Semen samples

Fifty-nine frozen semen samples taken from dairy bulls, were obtained from four companies in Brazil. The samples were thawed in a water bath at 37 °C for 30 s and centrifuged at 1200 × *g* for 10 min to separate the sperm cells from the seminal plasma and diluents. The obtained cell pellet was washed twice with PBS (0.9% phosphate buffered saline, pH 7.4) and again centrifuged at 1200 × *g* for 10 min. Finally, the pellet was re-suspended in 200 μL of PBS for subsequent DNA extraction.

2.4. DNA extraction

Genomic DNA was extracted from each sample by using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions. Extracted DNA was quantified by using Nanovue (GE, Fairfield, CT, USA). The DNA quality was checked by bovine β-globin gene PCR, as described by Freitas et al. (2003).

2.5. Detection of viral DNA and genotyping

Viral DNA was amplified by PCR assays using Master Mix Promega kit (Promega, Fitchburg, WI, USA) following the manufacturer's instructions. Reactions were performed in a two-step process. First, all the DNA samples were screened for the presence of BPV DNA using the FAP59/64 consensus primers under the conditions described by Ogawa et al. (2004) with the modifications described by Carvalho et al. (2012). Second, the DNA samples were amplified using BPV type-specific primers (BPV2–5 and 8–10 for skin lesions and BPV2–5 for fluids), in compliance with the amplification protocol described by Carvalho et al. (2012). All the amplification products were visualized by 2% TAE agarose gel electrophoresis and subsequent ethidium bromide staining. The positive and negative controls are described by Carvalho et al. (2012). Amplicons obtained by FAP59/64 PCR and specific primers were sequenced to identify/confirm the viral type, respectively.

2.6. Identification of a putative new BPV type

The sample that tested positive for the presence of a putative new BPV type was once again amplified by PCR using a High Fidelity DNA polymerase (GE) and the degenerate primers outlined above for confirmation. The PCR products were cloned into pGEM-T vector (Promega, USA) followed by the transformation of competent DH5α bacteria. The bacterial clones were randomly selected for confirmation. At least two different positive clones were sequenced twice, in both directions, with an ABI 3100 Applied Biosystems DNA sequencer and Sanger BigDye terminator v 3.1 cycle sequencing kit.

Sequencing quality and contig assembly were carried out using Pregap4 and Gap4 programs (Staden, 1996). Only sequences with a Phred value above 30 were considered for the contig assembly. Local sequence alignments were carried out to determine sequence identity with BLAST (Altschul et al., 1990). A multiple sequence alignment was carried out using Muscle (Edgar, 2004) and ClustalW algorithms, incorporated in MEGA5 software (Tamura et al., 2011). The identity of nucleotide and amino acid sequences was determined by using BioEdit v. 7.1.3 software (Hall, 1999).

2.7. Phylogenetic analysis

Phylogenetic analysis was carried out with amino acid sequences of BPV types and putative novel types isolated from Northeastern Brazil, using the Maximum Likelihood method with WAG + G as an amino acid substitution model in PhyML 3.0 (Guindon et al., 2010). The tree topology was estimated by using the best solution from the Nearest Neighbor Interchange (NNI) and Subtree Pruning and Regrafting (SPR) methods. An initial BIONJ tree was used, and the taxa were randomly added. 1000 non-parametric bootstrap replicates were used to estimate the statistical support of the obtained branches.

3. Results

The total number of each BPV type that was amplified by the two PCR systems, is summarized in Table 1. The number of positive samples with BPV type-specific primers ranged from 95 to 100% while the number of positive samples with consensus primers ranged from 5 to 54%. The FAP59/64 consensus primers amplified a higher number of skin lesion samples (54%) than the fluid samples (5–7%) (blood and semen). Also, the BPV type-specific primers had a level of positivity that was similar in the two types of samples (100% for skin lesion and 95% for semen and blood).

In the case of skin lesions, when the BPV type-specific primers were used, 89% of the samples had co-infection. However, co-infection was not observed when the consensus primers were used. Furthermore, the BPV types 4 and 9 were not detected with the consensus primers, while the rates of prevalence of these BPV types with the use of specific primers were 25% (BPV4) and 3% (BPV9). The results of BPV prevalence are shown in Fig. 1. High discrepancies in the prevalence of BPV were found when the results of the BPV type-specific primers and consensus primers were compared. BPV2 showed a prevalence of 99% with BPV type-specific primers while only 16% of the samples were positive for BPV2 when consensus primers were used. The prevalence rate of BPV3 was 78% with type-specific primers and only 8% with consensus primers. In addition, BPV10 showed a high discrepancy in the prevalence rate when the two PCR systems were compared – it showed 33% of prevalence with type-specific primers while 12% of the samples were positive for BPV with consensus primers. When the BPV

Table 1
Number of samples positive for BPV DNA.

Samples	FAP 59/64	BPV type-specific
Skin lesions	39/72 (54%)	72/72 (100%)
Blood	3/60 (5%)	57/60 (95%)
Semen	3/40 (7%)	59/59 (100%)

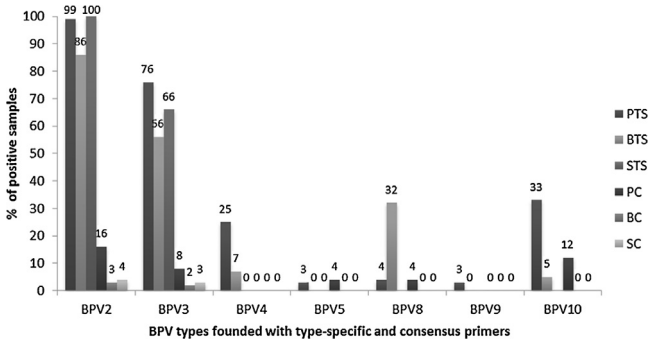

Fig. 1. BPV types amplified in lesion and fluid samples by two PCR systems. PTS means papilloma sample amplified with type-specific primers; BTS means blood sample amplified with type-specific primers; STS means semen sample amplified with type-specific primers; PC means papilloma sample amplified with consensus primers; BC means blood sample amplified with consensus primers; SC means semen samples amplified with consensus primers.

type-specific primers were used for semen, 66% of the samples showed co-infection while 49% of the blood samples had co-infection. As demonstrated in the skin samples, co-infection was not observed with the use of the consensus primers. BPV2 and 3 had the highest prevalence rate in all kinds of samples (skin, semen and blood).

The consensus primers showed a wide range of BPVs types and putative new types. Fourteen different BPV types and subtypes were found in the samples (BPV1–6, BPV8–10, BAPV3, BAPV10, BRUEL3–5) while 7 out of 7 possible BPV types tested with BPV type-specific primers were found. In the sample amplified with consensus primers, 50% showed a new putative BPV type described previously. Furthermore, the use of consensus primers allowed the detection of some new putative BPV types. Nucleotide identity analysis showed that one isolate (BPV/UFPE03BR) is an unreported putative BPV type. Another isolate (BPV/UFPE05BR) found in this study is a new BPV11 subtype (Fig. 2). The identity between the BPV/UFPE03BR sequence and BPV6 L1 sequence was 71.5%. This suggests that BPV/UFPE03BR isolate is a novel BPV type. The identity between the BPV/UFPE05BR sequence and BPV11 L1 sequence

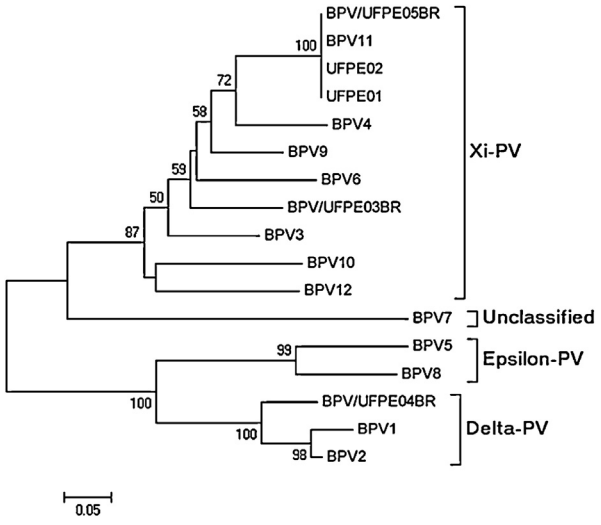

Fig. 2. Maximum likelihood phylogenetic tree of bovine papillomaviruses based on partial sequences of L1 ORF, which comprises 12 BPV types and isolates from Brazil. Three groups of viruses are distinguished, which forms the previously described genera (*Xipapillomavirus*, *Deltapapillomavirus* and *Epsilonpapillomavirus*). Unclassified isolates are also presented. Numbers in the nodes are bootstrap support values of the branches determined by 1000 replicates, and the values below 50% are not shown.

was 98%, which indicates that it is a new BPV11 subtype. These two nucleotide sequences were deposited at GenBank under accession numbers: JQ897974 and JQ897976.

The phylogenetic tree confirmed that the isolate BPV/UFPE03BR belongs to a new viral type, with 59% of confidence based on bootstrap (Fig. 2). This isolate is close related to all *Xipapillomavirus* genus members, which indicates that it belongs to this genus. The BPV/UFPE05BR isolate was clearly associated with BPV11, which confirms that it is a new subtype. The majority of the branches were statistically well supported with a confidence level of at least 50%. In addition, the clade that corresponded to *Xipapillomavirus* genus was strongly supported (Fig. 2).

4. Discussion

This study compares two PCR strategies for BPV detection and characterization. Knowledge of BPV diversity and epidemiology is of crucial importance for establishing prevention strategies and understanding the evolution of this group of viruses. Thus, the employment of an accurate PCR strategy is necessary to obtain reliable data to increase the knowledge of BPV biology.

This study shows that the FAP59/64 consensus primers had a lower level of sensitivity than BPV type-specific primers. In a previous study that employed consensus and type-specific primers for HPV detection, it was found that the sensitivity of type-specific primers was higher than the consensus primers (Qu et al., 1997). Besides, FAP primers have been used to amplify BPV DNA (Antonsson and Hansson, 2002; Ogawa et al., 2004; Claus et al., 2007; Carvalho et al., 2012). However, these primers were designed to amplify an HPV L1 region (Forslund et al., 1999). Thus, the mismatches between the consensus primer sequences and BPV L1 sequences could lower the sensitivity of these sets of primers. Qu et al. (1997) stated that the efficiency of consensus primers may be related to the number, position and stability of the mismatch. Furthermore, these authors confirmed that there were differences in type-specific amplification efficiency which could be attributed to a degeneracy synthesis in the consensus primers. There are three mismatches between FAP primers and BPV2, and there are six, four and five mismatches between the FAP primers and BPVs 3, 4 and 10, respectively. This high number of mismatches could help to explain the low sensitivity of FAP primers when compared with BPV type-specific primers.

Despite this, consensus primers enable the amplification of a high number of BPV types, including types that remain uncharacterized. In this study, one putative new BPV type and one novel BPV11 subtype were found by using this PCR system. Previous studies that involved consensus primers detected about 31 putative new BPV types (Forslund et al., 1999; Antonsson and Hansson, 2002; Ogawa et al., 2004; Maeda et al., 2007; Claus et al., 2008; Carvalho et al., 2012). There may be an underestimation of the extent of the spread of BPV. Only 13 BPV types have been described so far, despite the great diversity found in HPV. In this context, the use of consensus primers is very important to increase the knowledge of BPV diversity.

In this study, a new putative BPV type that belongs to *Xipapillomavirus* genus and a novel BPV11 subtype were described. These results suggest that there is a wide diversity of BPV types that infect cattle, and an understanding of this diversity is necessary for therapeutic treatment and to improve diagnostic methods. Recent studies have described novel BPV types, and thus demonstrated the extent of this diversity (Claus et al., 2008; Hatama et al., 2008, 2011; Carvalho et al., 2012; Zhu et al., 2011; Lunardi et al., 2013).

The presence of novel putative BPVs in Brazilian cattle suggests that other uncharacterized BPV types could be infecting cattle worldwide. The use of consensus primers is very important in the

investigation of novel PV types, and shows the need for this kind of approach. Further studies aimed at detecting and characterizing novel PV types and their variants are required to obtain a better understanding of their biology and their association with other pathological factors.

In conclusion, both FAP and BPV type-specific primer sets amplified a wide range of BPV types in skin lesions, blood and semen samples. However, BPV type-specific primers were more sensitive than consensus primers and it was thus possible to detect co-infection of different BPV types in the samples. On the other hand, the consensus primers are a very suitable means of detecting novel BPV types and subtypes, which is also important. Hence, the choice of the PCR primer system plays an important role in epidemiological investigations of BPV. When undertaking a more complete study, it should be noted that both systems are complementary.

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